Chondrocytes and chondrons for tissue engineering of cartilage

Vonk, L.A.

2010

CHAPTER 4

CONTRACTION-INDUCED MMP13 AND -14 EXPRESSION BY GOAT ARTICULAR CHONDROCYTES IN COLLAGEN TYPE I BUT NOT TYPE II GELS

Agnes D. Berendsen[1], Lucienne A. Vonk[1], Behrouz Zandieh Doulabi[2], Vincent Everts[1], and Ruud A. Bank[3]

[1]Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Amsterdam, The Netherlands. [2]Department of Orthopaedic Surgery, VU University Medical Center, Amsterdam, The Netherlands. [3]Stem Cell and Tissue Engineering Research Group, Medical Biology Section, University Medical Center Groningen, Groningen, The Netherlands

Submitted

ABSTRACT

Collagen gels are promising scaffolds to prepare an implant for cartilage repair but several parameters as collagen concentration and composition as well as cell density should be carefully considered as they are reported to affect phenotypic aspects of chondrocytes. In this study we investigated whether contraction of collagen gels affects the expression of matrix metalloproteinases (Mmps) by goat articular chondrocytes in gels composed of collagen type I or type II. Only floating collagen I gels and not those attached or composed of type II collagen contracted during a culture period of 12 days. The gels were reduced to 35% of the initial surface area and under these conditions an upregulation was found of both Mmp13 and -14 gene expression, whereas Mmp1 expression was not affected. The release of hydroxyproline in the culture media indicating matrix degradation was significantly increased in collagen I gels (contraction) compared to collagen II gels (no contraction). Furthermore, blocking contraction of floating collagen I gels by cytochalasin B inhibited Mmp13 and -14 expression and hydroxyproline release. We conclude that the collagen composition of the gels affects matrix contraction by articular chondrocytes and that contraction induces an increased Mmp13 and -14 expression as well as matrix degradation.

INTRODUCTION

Natural healing of articular cartilage defects generally does not occur and numerous strategies have emerged to repair or replace the damaged structures[1]. Recent research efforts have focused on tissue engineering as a promising approach for cartilage regeneration and repair. Tissue engineering is a multidisciplinary research area that incorporates both biological and engineering principles for the purpose of generating new, living tissues to replace the diseased/damaged tissue and restore tissue/organ function. The need for improved treatments has motivated research aimed at creating new cartilage that is based on collagen-glycosaminoglycan templates[2], isolated chondrocytes[3,4], and chondrocytes attached to natural (collagen, alginate, agarose, glycosaminoglycan)[5] or synthetic (poly(L-lactic acid) (PLA), poly(glycolic acid)) (PGA) polymers[6-8].

Collagen gels are frequently used scaffolds to study chondrocyte behavior[9-15]. Such behavior was reported to be affected by the chemical composition of the gels. Studies comparing type I and type II collagen gels showed increased rates of cytokine-regulated proliferation and proteoglycan synthesis by the chondrocytes in the type II gels[9,16]. Also in collagen-glycosaminoglycan (GAG) sponge-like matrices chondrocytes better maintained their spherical morphology and had greater biosynthetic activity during culture if matrices were composed of collagen type II compared to those consisting of collagen type I[17,18]. Despite the predominance of cells with the chondrocyte morphology in the type II scaffolds, both the type I and type II collagen-GAG matrices underwent cell-mediated contraction[19]. Chondrocyte-mediated contraction of the matrices is a serious drawback for the application of such scaffolds for articular cartilage tissue engineering. *In vivo* deformation of the matrix could result in a loss of contact between the implanted device and the host tissue, thereby decreasing the chances for successful integration of the repair tissue. Previous studies have also reported the contraction *in vitro* of other types of scaffolds by articular chondrocytes: fibrin gels[20,21], PGA scaffolds[22,23], and collagen gels[24,25]. Nehrer et al.[17] reported that shrinkage of collagen-GAG sponges was observed to a much greater extent in type I collagen containing material compared to those composed of type II collagen.

In addition to the effects of the chemical composition of the gels on the chondrocyte phenotype, cell-mediated contraction was also shown to affect the cellular activity in the gels. Synthesis of cartilage-specific type II collagen was observed in contracted collagen-GAG matrices, whereas different types of cross-linking decreased both the amount of matrix contraction and matrix production[26]. Furthermore, the ratio of Col2a1/ Col1a2 mRNA and also of α10/ α11 integrin mRNA produced by the chondrocytes was better maintained in contracting collagen I gels (floating gels) compared to non-contracting collagen I gels (attached gels)[25]. However, expression of the collagenases matrix metalloproteinase (Mmp) 1 and -13 by the chondrocytes was also increased in the contracted collagen I gels possibly resulting in overall matrix degradation and instability.

Use of chondrocyte-seeded collagen matrices for articular cartilage engineering requires that both chemical and biomechanical matrix properties that are known to influence the chondrocyte phenotype need to be carefully controlled. So far it is not clear whether the collagen composition of the gels affects matrix contraction by the chondrocytes possibly leading to changes in matrix synthesis and degradation. In this study we investigated whether the presence of collagen type I or type II affects matrix contraction by goat articular chondrocytes and/or expression levels of matrix proteins, Mmps and the subsequent degradation of collagen.

MATERIALS AND METHODS

Cell isolation

Chondrocytes were isolated from the articular cartilage of five skeletally mature female Dutch milk goats. Articular cartilage slices were obtained from one hindlimb joint and collected in 15 ml serum free DMEM (Gibco BRL, Paisley, Scotland) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B; Gibco BRL). The cells were obtained using a sequential digestion with 2.5% (w/v) Pronase E (Sigma Chemical Co., St. Louis, MO) in DMEM with antibiotics for 60 min at 37°C and then with 0.125% (w/v) Liberase blendzyme 3 (Roche Diagnostics, Mannheim, Germany) in DMEM with 25% FCS (HyClone, Logan, UT) and antibiotics overnight at 37°C. After filtration of the suspension through a 70 µm cell strainer (BD Biosciences, Bedford, MA) to remove undigested cartilage, cells were washed twice with 20 ml culture medium (DMEM + 10% FCS + antibiotics). Chondrocytes were then suspended in culture medium and the number of cells in a sample of the cell suspension was counted in a Bürker-Türk chamber. Only freshly isolated cells were used for the separate experiments (no passaging).

Preparation of collagen gels

Collagen gels were composed of either rat tail collagen type I (BD Biosciences) or chicken sternum collagen type II (Sigma Chemical Co., St. Louis, MO). The final collagen concentration in the gels was 2 mg/ml; a concentration whereby the chondrocytes were kept within the gels and did not sink towards the bottom of the wells.

A collagen mixture was prepared on ice and contained per ml: 100 µl MEM 10×, 800 µl collagen (2.5 mg/ml in 0.02 N acetic acid), 6 µl 5 M NaOH and 100 µl cell suspension (1×10^6 or 1×10^7 cells/ml). The solution was mixed thoroughly and per well of a 96-wells plate (Costar, Cambridge, MA) 100 µl was added. These gels attached to the wall and bottom of the well and represented the attached gels. To get floating (non-attached) gels, wells were coated with 1% BSA (Sigma) in PBS for 30 minutes prior to the preparation of the gels and the wells were allowed to dry under sterile conditions. The collagen gel lattices were formed when the plates were placed in a humidified incubator at

37°C in an atmosphere of 5% CO_2 for 60 minutes. After this period, 175 µl DMEM supplemented with 10% FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B) was added to each gel. The culture medium was changed every two days and pooled per culture condition. During the study, unseeded gels were kept in culture as controls. At regular time intervals the gels were micro graphed and the surface area of the gels was measured by the use of the Leica Qwin program and expressed as a % of the initial surface area.

To determine the effect of contraction, media were supplemented with 10 µM cytochalasin B (Sigma)[25]. Cytochalasin B was first dissolved in DMSO in a 1000x stock solution which was then added to the collagen gels harboring chondrocytes (final concentration of DMSO in the medium was 0.1%). Cytochalasin B was present during the entire culture period.

RNA analysis and quantitative real time PCR

Total RNA from cells cultured in the separate gels (N=4) was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA concentration was measured with the NanoDrop (Nanodrop Technologies, Wilmington, DE). The reverse transcriptase reaction was performed using 750 ng total RNA, 250 U/ml Transcriptor Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany), 0.08 U random primers (Roche Diagnostics), and 1 mM of each dNTP (Invitrogen) in Transcriptor RT reaction buffer at 42°C for 60 min followed by an inactivation of the enzyme at 70°C for 10 min. Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics); the LightCycler reactions were prepared in 20 µl total volume with 7 µl PCR-H_2O, 0.5 µl forward primer (0.2 µM), 0.5 µl reverse primer (0.2 µM), 10 µl LightCycler Mastermix (LightCycler 480 CYBR Green I Master; Roche Diagnostics), to which 2 µl of 4 times diluted cDNA was added as PCR template. Primers (Invitrogen) used for real-time PCR are listed in Table 1. Specific primers were designed from sequences available in the data banks, based on homology in conserved domains between human, mouse, rat, dog and cow. The amplified PCR fragment extended over at least one exon-border, except for 18S. 18S, tyrosine 3-monooxygenase (Ywhaz) and hypoxanthine phosphoribosyltransferase 1 (Hprt1) were used as housekeeping genes and the gene expression levels were normalized for the normalization factor calculated with the equation $\sqrt[3]{(18S \times Ywhaz \times Hprt)}$. Expression of the housekeeping genes was not affected by the experimental conditions, *e.g.* the collagen composition of the gels or the presence of cytochalasin B in the culture media. With the LightCycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by LightCycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

Table 1. Primer sequences used for real time PCR

Target gene		Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)
18S	Forward	5' GTAACCCGTTGAACCCCATT 3'	57	151
	Reverse	5' CCATCCAATCGGTAGTAGCG 3'		
Acan	Forward	5' CAACTACCCGGCCATCC 3'	57	160
	Reverse	5' GATGGCTCTGTAATGGAACAC 3'		
Col1a1	Forward	5' TCCAACGAGATCGAGATCC 3'	57	191
	Reverse	5' AAGCCGAATTCCTGGTCT 3'		
Col2a1	Forward	5' AGGGCCAGGATGTCCGGCA 3'	56	195
	Reverse	5' GGGTCCCAGGTTCTCCATCT 3'		
Ddr2	Forward	5' CCTCTGGCATGAAGTACCT 3'	57	341
	Reverse	5' GAGAGTTCTTCCGAGACCAA 3'		
Hprt1	Forward	5' GCTGACCTGCTGGATTACAT 3'	56	260
	Reverse	5' CTTGCGACCTTGACCATCT 3'		
Itgα1	Forward	5' AGGACAGTGCCTATAACACC 3'	56	234
	Reverse	5' CGCTGTCACTTGTTGCACTT 3'		
Itgα2	Forward	5'-ACAGACAAGGCTGGTGACA-3'	60	258
	Reverse	5'-TGCCGTTAGCTGTACTGTCT-3'		
Itgα6	Forward	5'-TGTACAGCTGCGACATCAC-3'	57	302
	Reverse	5'-CTCATGGCCTCTCAATC-3'		
Itgα10	Forward	5'-TCCGAACGAAGGAAGAAGTG-3'	56	253
	Reverse	5'-GGAAGCTCCTCTCCATCAT-3'		
Itgα11	Forward	5'-TCGTGCTCCACAGCTGAAT-3'	55	210
	Reverse	5'-TCCTCCTCACGGAAGATGAA-3'		
Itgβ1	Forward	5'-ACACCAGCTAAGCTCAGGAA-3'	56	230
	Reverse	5'-ACCAGCAGCCGTGTAACAT-3'		
Mmp1	Forward	5'-GTGGACCATGCCATTGAGAA-3'	55	387
	Reverse	5'-GGCTTGGATGCCATCAATGT-3'		
Mmp13	Forward	5' GGAGCATGGCGACTTCTAC 3'	56	208
	Reverse	5' GAGTGCTCCAGGGTCCTT 3'		
Mmp14	Forward	5' CAGAGATCAAGGCCAATGTTC 3'	56	206
	Reverse	5' CTCACGGATGTAGGCATAGG 3'		
Ywhaz	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	56	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'		

Acan, aggrecan; Col1a1, α1(I)procollagen; Col2a1, α1(II)procollagen; Ddr2, Discoidin domain receptor 2; Hprt1, hypoxanthine phosphoribosyltransferase 1; Itgα1,2,6,10, and 11, integrin α1,2,6,10, and 11; Itgβ1, integrin β1; Mmp1,13, and 14, matrix metalloproteinase 1, 13, and 14; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide.

Hydroxyproline assay

The amount of hydroxyproline released into the culture medium was determined using a modified colorimetric assay[27].

Statistical analysis

Quantitative data were expressed as means ± S.D. Data were statistically analyzed using One-way Analysis of Variance (ANOVA) test followed by Tukey-Kramer's Multiple comparisons test or an unpaired t-test with Welch correction. Effects were considered statistically significant at $p<0.05$ (two-tailed).

RESULTS

Collagen composition of gel affects contraction, gene expression levels for MMP's and degradation of gels

We first studied the effect of gels composed of collagen type I or type II on matrix contraction and relative gene expression levels by goat articular chondrocytes. Chondrocytes were cultured in floating collagen gels for a period of 12 days (1×10^5 cells seeded per gel). Only collagen I gels seeded with chondrocytes contracted after culture for 7 days and the initial surface area of these gels was reduced by 65% after a culture period of 12 days (Fig. 1A). Contraction of collagen II gels seeded with chondrocytes and contraction of unseeded collagen I and collagen II gels did not occur. Even after a period up to 4 weeks of culture contraction of these gels was not seen (not shown).

Relative gene expression levels for Mmp1, Mmp13 and Mmp14 by the chondrocytes were analyzed after culture for 2, 6 and 12 days. The collagen composition of the gels strongly affected the Mmp expression levels. Mmp1 expression was significantly increased in collagen II gels both after 2 days (57-fold), 6 days (103-fold) and 12 days (8-fold) of culture compared to collagen I gels (Fig. 1B). Expression of Mmp13 was also significantly increased in collagen II gels after 6 days culture (4-fold). After 12 days, however, higher expression levels were observed in collagen I gels (19-fold) compared to collagen II gels (Fig. 1B). Mmp14 expression was significantly increased in collagen I gels compared to collagen II gels both after culture for 6 (4-fold) and 12 days (8-fold) (Fig. 1B).

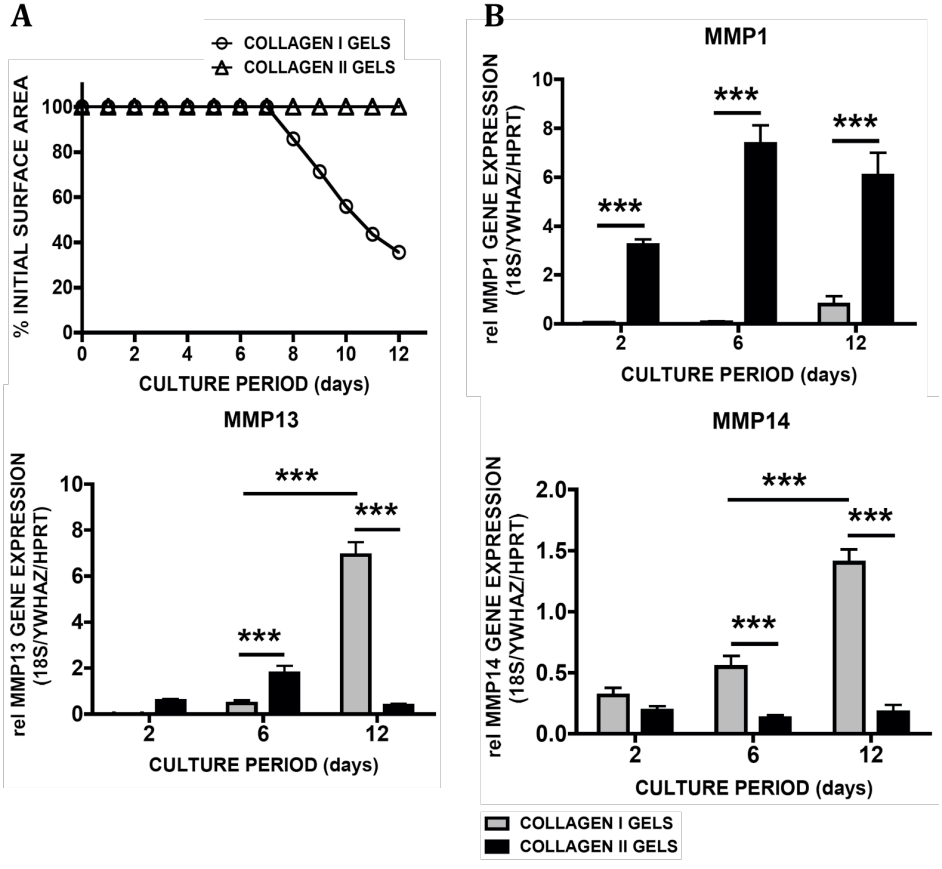
Changes in Mmp expression levels in the chondrocytes were also observed during matrix contraction. Although contraction of the collagen I gels had no effect on Mmp1 expression, Mmp13 and Mmp14 expression levels were upregulated (15-fold and 3-fold, respectively) compared to the non-contracted gels (Fig. 1B).

In addition to Mmp gene expression, we also analyzed expression for collagen type II (Col2a1), aggrecan and collagen type I (Col1a1) as markers of differentiated or dedifferentiated chondrocytes, respectively. The collagen composition of the gels appeared

to affect the expression levels for these matrix proteins. Expression of cartilage-specific proteins collagen type II and aggrecan was significantly upregulated 25-fold (Col2a1) and 5-fold (aggrecan) in collagen I gels compared to collagen II gels after culture for 2 days (Fig. 1C). At later time-points the relative expression of these proteins was not affected by the collagen composition but expression levels were decreased with expression of Col2a1 below 0.05 after 12 days culture (Fig. 1C). Col1a1 expression (a marker of dedifferentiation of chondrocytes) was significantly increased in collagen I gels compared to collagen II gels after culture for both 6 days (170-fold) and 12 days (7-fold) (Fig. 1C). In addition to the collagen composition of the gels, matrix contraction also influenced relative expression levels for the matrix proteins. Contraction of collagen I gels resulted in downregulation of the expression of Col2a1, aggrecan and Col1a1 by the chondrocytes.

We then assessed whether the collagen composition of the gels and/or matrix contraction affected the degradation of collagen (both initially present in the scaffold as well as possibly newly synthesized by the chondrocytes) by assessing the release of hydroxyproline in the culture media by the chondrocytes during a culture period of 12 days. Hydroxyproline concentrations were significantly higher of contracted collagen I gels compared to non-contracted collagen II gels (Fig. 1D).

Fig. 1



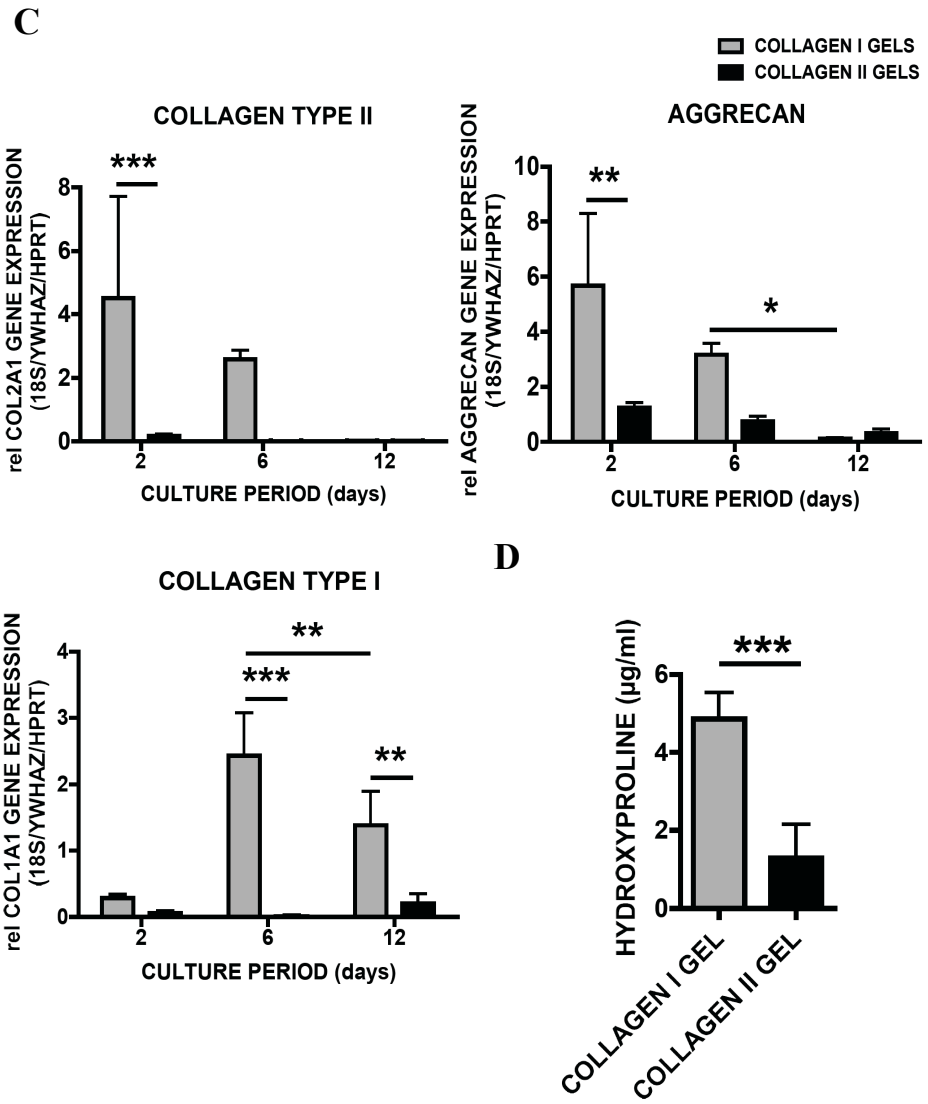


Figure 1: *Effect of collagen composition of gels on contraction (A), relative gene expression levels for Mmps (B) and ECM proteins (C), and release of hydroxyproline in the culture media (D) by goat articular chondrocytes cultured in floating collagen I or collagen II gels. The gels were seeded with 1.0×10^5 cells per 100 µl gel and cultured for 12 days. (A) Contraction is expressed as a % of the initial surface area. Values represent the mean surface area (n=4). Gels composed of collagen type I contracted during the culture period, which was not observed for gels composed of collagen type II. Statistical analysis: the difference in the % initial surface area of collagen I gels versus collagen II gels was significant ($p<0.001$) after a culture period of 8 days and at later time-points. (B, C) Data are normalized to 18S, Ywhaz and Hprt expression. Values represent the mean relative expression ± S.D. (n=4) with *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Gene expression levels were affected by the collagen composition of the gels. (D) Hydroxyproline concentrations (µg/ml) released into the culture media after culturing chondrocytes in collagen I or collagen II gels for a period up to 12 days.*

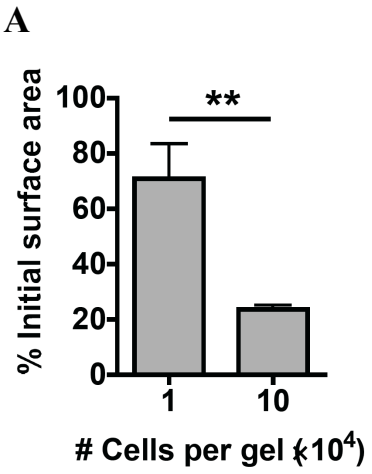
Cell seeding density affects contraction and gene expression levels for MMP's

As changes in Mmp expression levels coincided with both the collagen composition of gels and with matrix contraction, we studied the effect of contraction only on Mmp expression. The extent of collagen gel contraction is related to the initial chondrocyte density[25] and therefore floating collagen I gels were seeded with 1×10^4 or 1×10^5 cells per gel at the start of the culture period. Contraction of gels seeded with 1×10^5 cells started after 6 days of culture and the initial surface area of these gels was significantly reduced by 76% after 9 days culture (Fig. 2A). Gels seeded with 1×10^4 cells per gel contracted after 8 days of culture which resulted in a 29% reduction of the initial surface area after 9 days (Fig. 2A). Thus, contraction of gels seeded with 1×10^5 cells was significantly higher compared to gels seeded with 1×10^4 cells.

Relative gene expression levels for Mmp1, Mmp13 and Mmp14 by the chondrocytes in the gels were analyzed after the culture period of 9 days. A high level of contraction (76% reduction of initial surface area) resulted in a significant upregulation of expression levels of Mmp1 (10-fold), Mmp13 (10-fold) and Mmp14 (2-fold) compared to a low level of contraction (29% reduction of initial surface area) (Fig. 2B).

In addition to Mmp expression, we also analyzed relative expression levels for Col2a1 and Col1a1 by the chondrocytes in the gels after the culture period of 9 days. A high level of contraction decreased the expression of both Col2a1 (6-fold) and Col1a1 (2-fold) compared to a low level of contraction (Fig. 2C).

Fig. 2



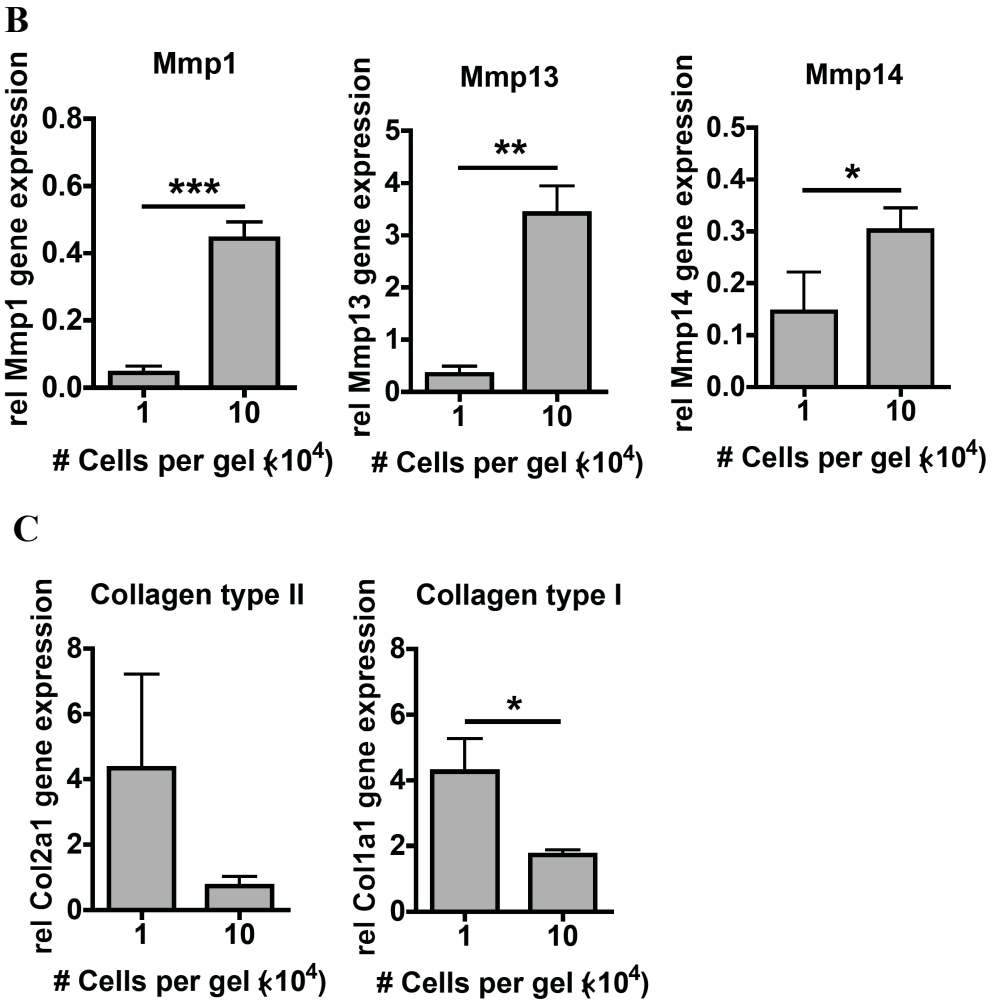


Figure 2: *Effect of different cell seeding densities on contraction (A) and relative gene expression levels (B) by goat articular chondrocytes in floating collagen I gels after culture for 9 days. The gels were seeded with 1.0×10^4 or 1.0×10^5 cells per 100 µl gel and cultured for 12 days. (A) Contraction is expressed as a % of the initial surface area after a 9 day culture period. Values represent the mean surface area ± S.D. (n=4) with ***p<0.001. Contraction of gels was dependent on the initial cell seeding density. (B) Data are normalized to 18S, Ywhaz and Hprt expression. Values represent the mean relative expression ± S.D. (n=4) with *p<0.05, **p<0.01 and ***p<0.001. Gene expression levels were affected by the initial cell seeding density after a culture period of 9 days.*

Cytochalasin B inhibits contraction, gene expression levels for MMP's and degradation of collagen

As the cell density may also affect gene expression levels, we analyzed whether the effects on Mmp expression were indeed depending on matrix contraction by assessing the expression levels following inhibition of contraction of gels. Chondrocytes were cultured in floating or attached collagen I gels (1×10^5 cells seeded per gel) with or without 10 μM cytochalasin B (CB). Only floating gels without CB contracted after a culture period of 6 days (Fig. 3A). The initial surface area of these gels was significantly reduced by 70% after culture for 12 days (Fig. 3A).

Relative gene expression levels for Mmp1, Mmp13 and Mmp14 were analyzed after culture for 2, 6 and 12 days. Mmp1 expression was significantly increased in floating gels without CB (12-fold) compared to floating gels with CB and in attached gels without CB (9-fold) compared to attached gels with CB after culture for 6 days (Fig. 3B). No differences in Mmp1 expression were measured in the gels after 12 days culture. Mmp13 expression was significantly upregulated in floating gels without CB compared to floating gels with CB after culture for 6 days (11-fold) and 12 days (45-fold). Mmp13 expression levels were also increased in attached gels without CB compared to attached gels with CB after culture for 6 days (14-fold) and 12 days (16-fold) (Fig. 3B). Mmp14 expression was significantly upregulated in floating gels without CB (5-fold) and attached gels without CB (3-fold) compared to floating and attached gels with CB after 12 days culture (Fig. 3B). In addition, contraction of floating gels without CB significantly increased Mmp13 and Mmp14 expression levels (2-fold and 3-fold, respectively).

Relative expression levels for Col2a1 and Col1a1 by the chondrocytes in the gels were also analyzed after culture for 2, 6 and 12 days. Col2a1 expression was significantly increased 2-fold in floating gels with CB compared to floating gels without CB and attached gels with or without CB after culture for 2 days (Fig. 3C). At later time-points the expression of Col2a1 was significantly upregulated in floating gels with CB 8-fold (6 days) and 273-fold (12 days) compared to floating gels without CB and in attached gels with CB 7-fold (6 days) and 132-fold (12 days) compared to attached gels without CB. Expression levels of Col1a1 were increased 224-fold in floating gels without CB compared to floating gels with CB and 1,039-fold in attached gels without CB compared to attached gels with CB after culture for 12 days (Fig. 3C). At this time-point the expression was significantly increased (6-fold) in attached gels without CB (no contraction) compared to floating gels without CB (contraction).

We then determined whether matrix contraction affected collagen degradation. Hydroxyproline concentrations were increased in floating gels without CB (contraction) compared to both floating gels with CB (no contraction) as well as attached gels without CB (no contraction) after a culture period of 12 days (Fig. 3D).

We studied the possible involvement of chondrocyte receptors for matrix proteins in the contraction-induced Mmp expression. Relative gene expression levels for integrin subunits and the discoidin domain receptor type-2 (Ddr-2) by the chondrocytes in floating

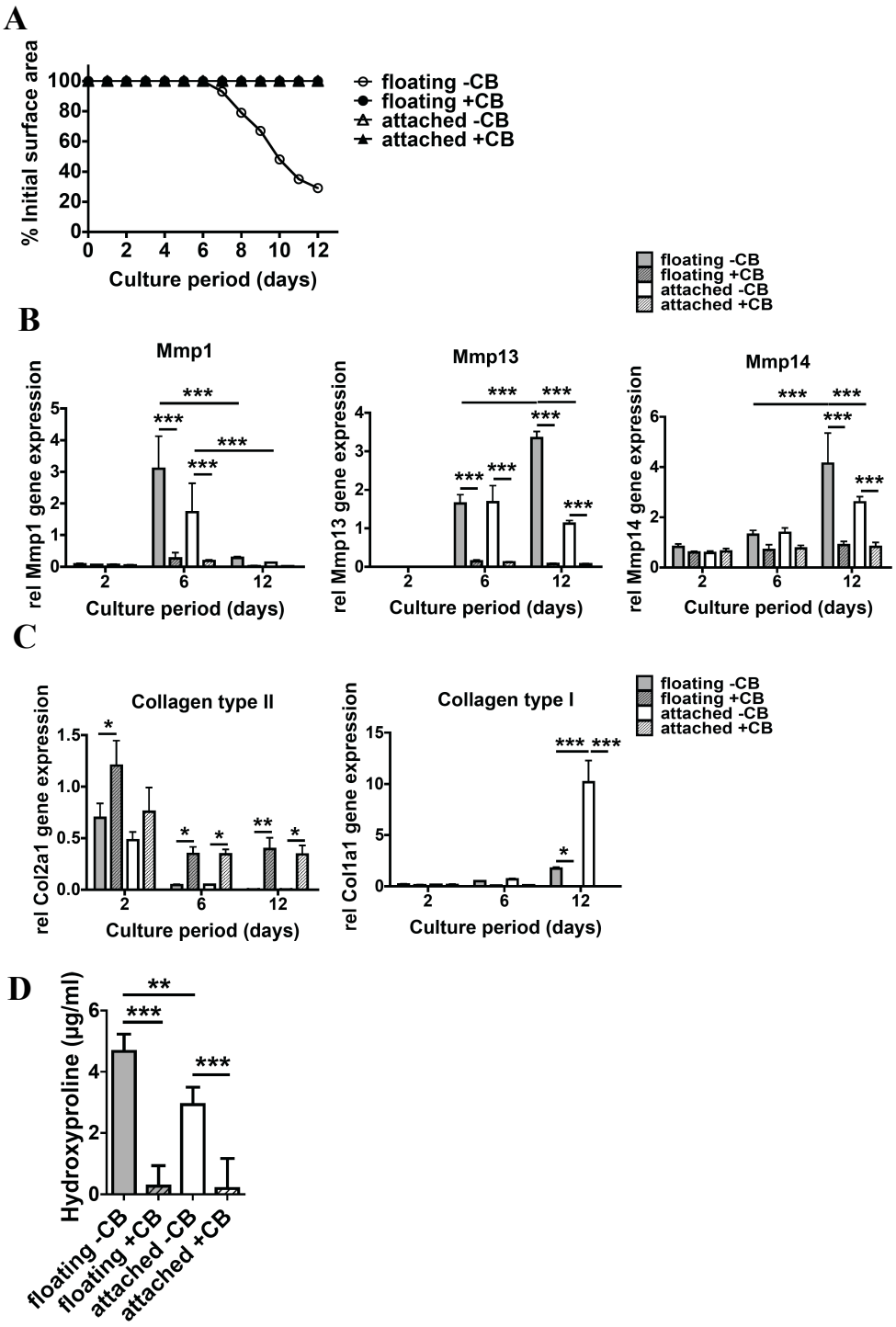
and attached gels with and without CB were analyzed after culture for 2, 6 and 12 days. Expression of integrin α1 was downregulated 3-fold in contracting gels (without CB) compared to non-contracting floating gels (with CB) after culture for 12 days (Fig. 3E). Integrin α10 expression levels were significantly increased in floating gels with CB 5-fold (6 days) and 7-fold (12 days) compared to floating gels without CB and in attached gels with CB 4-fold (6 days) and 7-fold (12 days) compared to attached gels without CB (Fig. 3E). Gene expression levels for integrins α2, α6 and α11 were hardly detected during the culture period analyzed (not shown). Integrin β1 expression was significantly increased 2-fold in attached gels without CB (6 days) compared to gels with CB and 3-fold in both floating and attached gels without CB compared to gels with CB (12 days) (Fig. 3E). Expression of Ddr-2 was not affected in either floating or attached gels with or without CB (Fig. 3E).

DISCUSSION

In the present study we found that floating collagen I gels but not collagen II gels are contracted by goat articular chondrocytes. This appeared to coincide with an upregulation of Mmp13 and -14 expression levels and increased collagen degradation in the gels. Such effects were not observed when contraction was prevented by use of attached collagen I gels or floating collagen I gels in the presence of cytochalasin B (CB). Interestingly, our study shows that matrix contraction thus not only affects gene expression levels in the chondrocytes, but it also results in the actual breakdown of collagens in the matrix.

The difference in contraction of gels composed of collagen type I or type II by the chondrocytes is intriguing. Collagen I gels contracted within several days of culture whereas contraction of collagen II gels was not observed even after a culture period of 4 weeks. Possible explanations for this observation could be related to the properties of the different collagen matrices, such as stiffness or cross-linking of collagens. Alternatively, the contractile activity of the chondrocytes in the collagen network may be related to the type of collagen the cells interact with. Such a collagen type-dependent effect was previously reported for matrix contraction by fibroblasts in the presence of collagen type V[28]. Under these conditions relatively low levels of this collagen type strongly enhanced matrix contraction by the cells. In contrast to our study, however, others have found contraction of chondrocyte-seeded collagen II matrices[18,19]. Yet, considerable differences in the culture conditions exist between our and the latter studies which may affect matrix

Fig. 3


A
% Initial surface area
Culture period (days)
floating -CB
floating +CB
attached -CB
attached +CB
B
Mmp1
Mmp13
Mmp14
rel Mmp1 gene expression
rel Mmp13 gene expression
rel Mmp14 gene expression
C
Collagen type II
Collagen type I
rel Col2a1 gene expression
rel Col1a1 gene expression
D
Hydroxyproline (µg/ml)

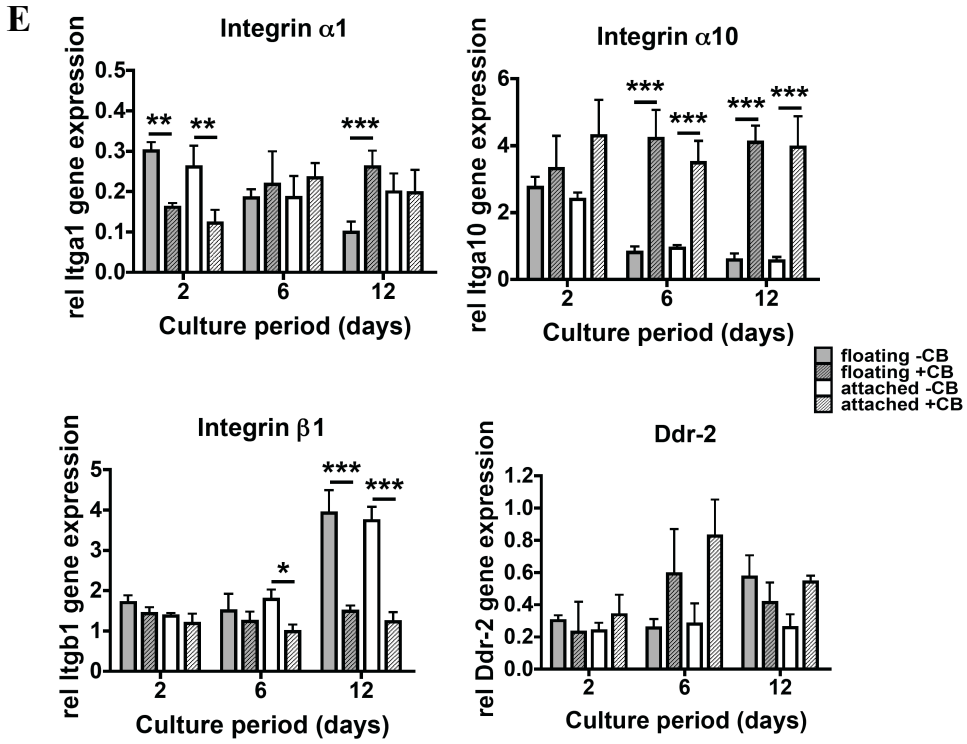


Figure 3: *Effect of the presence of cytochalasin B (CB) on contraction (A), relative gene expression levels for Mmps (B), ECM proteins (C), chondrocyte receptors (E), and release of hydroxyproline in the culture media (D) by goat articular chondrocytes in floating or attached collagen I gels. The gels were seeded with 1.0×10^5 cells per 100 µl gel and cultured for 12 days with or without 10 µM CB. (A) Contraction is expressed as a % of the initial surface area. Values represent the mean surface area (n=4). Floating gels without CB contracted during the culture period, which was not observed for floating gels with CB or attached gels with or without CB. Statistical analysis: the difference in the % initial surface area of floating gels without CB versus floating gels with CB and attached gels with or without CB was significant ($p<0.05$) after a culture period of 8 days and at later time-points ($p<0.001$). (B, C, D) Data are normalized to 18S, Ywhaz and Hprt expression. Values represent the mean relative expression ± S.D. (n=4) with *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Gene expression levels were affected by culture of the chondrocytes in the different types of gels, floating or attached, with or without the presence of CB.*

contraction, including collagen concentration in the matrices, initial cell seeding density and the presence of glycosaminoglycans.

The regulation of matrix-degrading protease activities in chondrocytes has been extensively studied. Mmps, ADAMTS4 and 5 and cysteine proteases are known to act in concert to degrade matrix components, including collagen type II[29]. Although we assume that the observed changes in Mmp expression levels in our study are related to the amount

of collagen degradation, the involvement of additional proteases can not be excluded. Of the Mmps, Mmp1, -2, -8, -13, and -14 were previously reported to have the capacity to cleave native, interstitial collagens[30-34]. We found that both the chemical (collagen composition) and biomechanical (contraction) properties of the matrix affected Mmp1, -13 and -14 expression, thus suggesting a tightly regulated mechanism controlling the expression of different Mmps. This mechanism likely involves the presence of various chondrocyte receptors that interact with both the matrix and the actin cytoskeleton as blocking contraction by use of attached gels or use of cytochalasin B to block the formation of actin filaments strongly inhibited Mmp expression. Several receptors were previously shown to be involved in Mmp13 expression by articular chondrocytes. Ronzière et al.[35] reported the involvement of integrin $\alpha1\beta1$ in the collagen induction of Mmp13 expression. A role for integrin $\alpha11\beta1$ was suggested in the expression of Mmp13 and -14 as culture of integrin $\alpha11$-deficient mouse embryonic fibroblasts in floating collagen gels resulted in inhibition of Mmp expression[36]. Apart from the integrins, expression of Mmp13 was induced by activation of discoidin domain receptor-2 (Ddr-2) associated with osteoarthritis in mice[37]. Conversely, in our study the expression levels of these receptors were not affected by matrix contraction thus suggesting the involvement of a different mechanism. As matrix contraction resulted in an upregulation of both Mmp13 and -14 expression levels, we hypothesize that contraction-induced Mmp13 expression may be mediated by the membrane-associated Mmp14. Mmp14 may be directly or indirectly connected to the F-actin cytoskeleton possibly via integrins or CD44[38]. Further studies are required to determine whether such mechanism is indeed involved in the observed effects.

Several studies reported a more pronounced chondrocyte phenotype in gels composed of collagen type II compared to collagen type I[9,16-18]. The synthesis of cartilage-specific proteins was also shown to be stimulated by contraction of floating collagen I gels by chondrocytes[25]. In contrast to these studies, our data reveal that expression of collagen type II and aggrecan was decreased in collagen II gels compared to those composed of type I. Furthermore, the expression of ECM proteins was decreased in contracted collagen I gels compared to non-contracted gels suggesting the involvement of cytoskeletal integrity in matrix synthesis. The chondrogenic phenotype was previously reported to be modulated by cell morphology and cytoskeletal organization, where disruption of the cytoskeleton resulted in maintenance of the phenotype[39-42]. In line with these studies, our data show that cytochalasin B treatment increases expression levels of both chondrogenic markers collagen type II and the integrin $\alpha10$-subunit. Interestingly, expression of the $\beta1$-integrin subunit was decreased although the heterodimer $\alpha10\beta1$ integrin is suggested to be a key receptor for collagen type II[43]. The differential effects of cytochalasin B on the expression of these integrin subunits may be explained by the fact that $\beta1$-integrin subunits interact with several α-subunits: integrin $\alpha1\beta1$ (a primary chondrocyte receptor for type II and type VI collagen) and $\alpha5\beta1$ (a primary chondrocyte fibronectin receptor) being the major ones for chondrocytes.

CONCLUSION

In this study we showed that both the chemical (collagen composition) as well as the biomechanical (contraction) properties of chondrocyte-seeded collagen gels not only affect the expression of cartilage-specific ECM proteins but also Mmp expression and matrix degradation. Chondrocytes are able to contract floating collagen I gels which coincides with an upregulation of Mmp13 and -14 expression and the actual degradation of collagen. These processes were not observed in type II collagen matrices. Conversely, the gene expression of (cartilage-specific) ECM proteins was less in collagen II gels compared to those composed of type I collagen. Furthermore, blockage of actin filament formation (by cytochalasin B) strongly increased the chondrocyte phenotype characterized by expression of collagen type II and integrin α10-subunit. Our data thus indicate that the collagen composition, floating or attached gels, and compounds affecting the F-actin cytoskeleton strongly affect matrix contraction, expression levels of ECM proteins and degradation by articular chondrocytes. Taking all these parameters into account may improve the quality of *in vitro* fabrication of tissue constructs for cartilage repair.

Acknowledgements

This study was supported by The Dutch Program for Tissue Engineering (DPTE, Grant # BGT 6734).

REFERENCES

1. Kuo CK, Wan-Ju L, Mauck RL, Tuan RS. Cartilage tissue engineering: its potential and uses. Curr Opin Rheumatol 2006;18:64-73
2. Stone KR, Rodkey WG, Webber RJ, McKinney L, Steadman JR. Future directions. Collagen-based prostheses for meniscal regeneration. Clin Orthop Relat Res 1990;252:129-35
3. Grande DA, Pitman MI, Peterson L, Menche D, Klein M. The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. J Orthop Res 1989;7:208-18
4. Takigawa M, Shirai E, Fukuo K, Tajima K, Mori Y, Suzuki F. Chondrocytes dedifferentiated by serial monolayer culture form cartilage nodules in nude mice. Bone Miner 1987;2:449-62
5. Wakitani S, Kimura T, Hirooka A, Ochi T, Yoneda M, Yasuï N, Owaki H, Ono K. Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. J Bone Jt Surg 1989;71:74-80
6. Vacanti C, Langer R, Schloo B, Vacanti JP. Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. Plast Reconstr Surg 1991;88:753-9
7. Von Schroeder HP, Kwan M, Amiel D, Coutts RD. The use of polylactic acid matrix and periosteal grafts for the reconstruction of rabbit knee articular defects. J Biomed Mater Res 1991;25:329-39
8. Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. J Biomed Mater Res 1993;27:11-23
9. Malemud CJ, Stevenson S, Mehraban F, Papay RS, Purchio AF, Goldberg VM. The proteoglycan synthesis repertoire of rabbit chondrocytes maintained in type II collagen gels. Osteoarthritis Cart 1994;2:29-42
10. Schuman L, Buma P, Verslegen D, de Man B, van der Kraan PM, van den Berg WB, et al. Chondrocyte behaviour within different types of collagen gel *in vitro*. Biomaterials 1995;16:809-14
11. Weiser L, Bhargava M, Attia E, Torzilli PA. Effect of serum and platelet-derived growth factor on chondrocytes grown in collagen gels. Tissue Eng 1999;5:533-44
12. Hunter CJ, Hunter SM, Malaviya P, Nereru RM, Leveriston ME. Mechanical compression alters gene expression and extracellular matrix synthesis by chondrocytes cultured in collagen I gels. Biomaterials 2002;20:1070-8
13. Chaipinyo K, Oakes BW, van Damme MPI. Effects of growth factors on cell proliferation and matrix synthesis of low density, primary bovine chondrocytes cultured in collagen I gels. J Orthop Res 2002;20:1070-8

14. Iwasa I, Ochi M, Uchio Y, Katsube X, Adachi N, Kaxasaki K. Effects of cell density on proliferation and matrix synthesis by chondrocytes embedded in atelocollagen gel. Artif Organs 2003;27:249-55
15. Ochi M, Uchio Y, Kwasaki K, Wakitani S, Iwasa I. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Jt Surg 2002;84(B):571-8
16. Qui W, Scully S. Extracellular collagens demonstrate a type specific influence on cytokine regulation of articular chondrocytes. Proc of the ORS, Atlanta, GA 1996;308
17. Nehrer S, Breinan HA, Ramappa A, Shortkroff S, Young G, Minas T, Sledge CB, Yannas IV, Spector M. Canine chondrocytes seeded in type I and type II collagen implants investigated *in vitro*. J Biomed Mater Res 1997;38:95-104
18. Veilleux NH, Yannas IV, Spector M. Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices *in vitro*. Tissue Eng 2004;10:119-27
19. Lee CR, Breinan HA, Nehrer S, Spector M. Articular cartilage chondrocytes in type I and type II collagen-GAG matrices exhibit contractile behavior *in vitro*. Tissue Eng 2000;6:555-65
20. Hunter CJ, Shieh AC, Nerem RM, Levenston ME. Effects of collagens I and II and chitosan on chondrocyte behavior in fibrin gel cultures. Sixth World Biomaterials Congress, Kamuela, Hawaii, 2000. p. 306
21. Shieh AC, Hunter CJ, Nerem RM, Levenston ME. Collagen induces contraction and inhibits matrix assembly by chondrocytes in fibrin gel cultures. 46th Annual Meeting on Orthopaedic Research Society, Orlando, FL, 2000. p. 917
22. Martin I, Vunjak-Novakovic G, Yang J, Langer R, Freed LE. Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Exp Cell Res 1999;253:681-8
23. Martin I, Suetterlin R, Baschong W, Heberer M, Vunjak-Novakovic G, Freed LE. Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation. J Cell Biochem 2001;83:121-8
24. Ohsawa S, Yasui N, Ono K. Contraction of collagen gel by the dedifferentiated chondrocytes. Cell Biol Int Reports 1982;6:767-74
25. Galois L, Hutasse S, Cortial D, Rousseau CF, Grossin L, Ronziere M-C, Herbage D, Freyria A-M. Bovine chondrocyte behaviour in three-dimensional type I collagen gel in terms of gel contraction, proliferation and gene expression. Biomaterials 2006;27:79-90

26. Lee CR, Grodzinsky AJ, Spector M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials 2001;22:3145-54
27. Creemers LB, Jansen DC, van Veen-Reurings A, van den Bos T, Everts V. Microassay for the assessment of low levels of hydroxyproline. Biotechniques 1997;22:656-8
28. Berendsen AD, Bronckers ALJJ, Smit TH, Walboomers XF, Everts V. Collagen type V enhances matrix contraction by human periodontal ligament fibroblasts seeded in three-dimensional collagen gels. Matrix Biol. 2006;25:515-22
29. Ruettger A, Schueler S, Mollenhauer JA, Wiederanders B. Cathepsins B, K, and L are regulated by a defined collagen type II peptide via activation of classical protein kinase C and p38 MAP kinase in articular chondrocytes. J Biol Chem 2008;283:1043-51
30. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific ¾- and ¼-length fragments. J Biol Chem 1995;270:5872-6
31. Seandel M, Noack-Kunnmann K, Zhu D, Aimes RT, Quigley JP. Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. Blood 2001;97:2323-32
32. Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M, Okada Y. Membrane type I matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J Biol Chem 1997;272:2446-51
33. Balbín M, Fueyo A, Knäuper V, López JM, Alvarez J, Sánchez LM, Quesada V, Bordallo J, Murphy G, López-Otín C. Identification and enzymatic characterization of two diverging murine counterparts of human interstitial collagenase (MMP-1) expressed at sites of embryo implantation. J Biol Chem 2001;276:10253-62
34. Gross J, Azizkhan RG, Biswas C, Bruns RR, Hsieh DS, Folkman J. Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxyprogesterone. Proc Natl Acad Sci USA 1981;78:1176-80
35. Ronzière M-C, Aubert-Foucher E, Gouttenoire J, Bernaud J, Herbage D, Mallein-Gerin F. Integrin α1β1 mediates collagen induction of MMP-13 expression in MC615 chondrocytes. Biochem Biophys Acta 2005;1746:55-64
36. Popova SN, Barczyk M, Tiger C-F, Beertsen W, Zigrino P, Aszodi A, Miosge N, Forsberg E, Gullberg D. α11β1 Integrin-Dependent Regulation of Periodontal Ligament Function in the Erupting Mouse Incisor. Mol Cell Biol 2007;27:4306-16
37. Xu L, Peng H, Wu D, Hu K, Goldring MB, Olsen BR, Li Y. Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. J Biol Chem 2005;280:548-55

38. Steffensen B, Häkkinen L, Larjava H. Proteolytic Events of Wound-healing-Coordinated Interactions among Matrix Metalloproteinases (MMPs), Integrins, and Extracellular Matrix Molecules. Crit Rev Oral Biol Med 2001;12:373-98
39. Lim YB, Kang SS, Park TK, Lee YS, Chun JS, Sonn JK. Disruption of actin cytoskeleton induces chondrogenesis of mesenchymal cells by activating protein kinase C-alpha signaling. Biochem Biophys Res Commun 2000;273:609-13
40. Woods A, Wang G, Beier F. RhoA/ROCK signaling regulates Sox9 expression and actin organization during chondrogenesis. J Biol Chem 2005;280:11626-34
41. Woods A, Beier F. RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. J Biol Chem 2006;281:13134-40
42. Connelly JT, Garcia AJ, Levenston ME. Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. J Cell Physiol 2008;217:145-54
43. Camper L, Hellman U, Lundgren-Akerlund E. Isolation, cloning, and sequence analysis of the integrin subunit alpha10, a beta1-associated collagen binding integrin expressed on chondrocytes. J Biol Chem 1998;273:20383-9